BARD Project Number: US-4323-10C

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Project Title: Molecular characterization and deployment of the high-temperature adult plant stripe rust resistance gene Yr36 (WKS1) from wheat

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Keywords: slow-rusting, resistance mechanism, alternative splicing, wild emmer wheat

LIST OF ABREVIATIONS
APX: Ascorbate peroxidase gene
GFP: Green fluorescent protein
HTAP: High temperature adult plant
PCR: Polymerase chain reaction
ROS: Reactive oxygen species
PSH: Puccinia striiformis f. sp. hordei
PST: Puccinia striiformis f. sp. tritici
RSL: Recombinant substitution line,
START: Steroidogenic acute regulatory protein-related lipid transfer domain
TE: Transposable element
WKS: Wheat Kinse START
WKS2-Ae: WKS2 gene derived from Aegilops comosa

Budget: IS: 118,000 $    US: 232,000 $    Total: 250,000 $

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Signature                        Signature
Principal Investigator           Authorizing Official, Principal Institution
Publications Summary (numbers)

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Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

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Abstract:

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* is one of the most destructive fungal diseases of wheat. Virulent races that appeared within the last decade caused drastic cuts in yields. The incorporation of genetic resistance against this pathogen is the most cost-effective and environmentally friendly solution to this problem. However, race specific seedling resistance genes provide only a temporary solution because fungal populations rapidly evolve to overcome this type of resistance. In contrast, high temperature adult plant (HTAP) resistance genes provide a broad spectrum resistance that is partial and more durable. The cloning of the first wheat HTAP stripe rust resistance gene *Yr36* (*Science* 2009, 323:1357), funded by our previous (2007-2010) BARD grant, provided us for the first time with an entry point for understanding the mechanism of broad spectrum resistance. Two paralogous copies of this gene are tightly linked at the *Yr36* locus (*WKS1* and *WKS2*).

The main objectives of the current study were to characterize the *Yr36* (*WKS*) resistance mechanism and to identify and characterize alternative *WKS* genes in wheat and wild relatives. We report here that the protein coded by *Yr36*, designated *WKS1*, that has a novel architecture with a functional kinase and a lipid binding START domain, is localized to chloroplast. Our results suggest that the presence of the START domain may affect the kinase activity. We have found that the *WKS1* was over-expressed on leaf necrosis in wheat transgenic plants. When the isolated *WKS1.1* splice variant transcript was transformed into susceptible wheat it conferred resistance to stripe rust, but the truncated variant *WKS1.2* did not confer resistance. *WKS1.1* and *WKS1.2* showed different lipid binding profiling. *WKS1.1* enters the chloroplast membrane, while *WKS1.2* is only attached outside of the chloroplast membrane. The ascorbate peroxidase (APX) activity of the recombinant protein of *TmtAPX* was found to be reduced by *WKS1.1* protein *in vitro*. The *WKS1.1* mature protein in the chloroplast is able to phosphorylate *TmtAPX* protein *in vivo*. *WKS1.1* induced cell death by suppressing APX activity and reducing the ability of the cell to detoxify reactive oxygen. The decrease of APX activity reduces the ability of the plant to detoxify the reactive *H₂O₂* and is the possible mechanism underlying the accelerated cell death observed in the transgenic plants overexpressing *WKS1.1* and in the regions surrounding a stripe rust infection in the wheat plants carrying the natural *WKS1.1* gene.

*WKS2* is a nonfunctional paralog of *WKS1* in wild emmer wheat, probably due to a retrotransposon insertion close to the alternative splicing site. In some other wild relatives of wheat, such as *Aegilops comosa*, there is only one copy of this gene, highly similar to *WKS2*, which is lacking the retrotransposon insertion. *WKS2* gene present in wheat and *WKS2-Ae* from *A.* showed a different pattern of alternative splice variants, regardless of the presence of the retrotransposon insertion. Susceptible Bobwhite transformed with *WKS2-Ae* (without retrotransposon insertion in intron10), which derived from *Aegilops comosa* conferred resistance to stripe rust in wheat. The expression of *WKS2-Ae* in transgenic plants is up-regulated by temperature and pathogen infection. Combination of *WKS1* and *WKS2-Ae* shows improved stripe rust resistance in *WKS1*×*WKS2-Ae* F₁ hybrid plants.

The obtained results show that *WKS1* protein is accelerating programmed cell death observed in the regions surrounding a stripe rust infection in the wheat plants carrying the natural or transgenic *WKS1* gene. Furthermore, characterization of the epistatic interactions of *Yr36* and *Yr18* demonstrated that these two genes have additive effects and can therefore be combined to increase partial resistance to this devastating pathogen of wheat.

These achievements may have a broad impact on wheat breeding efforts attempting to protect wheat yields against one of the most devastating wheat pathogen.
Achievements:

The main objectives of the current study were to characterize the $Yr36$ ($WKS$) resistance mechanism and to identify and characterize alternative $WKS$ genes in wheat and wild relatives. Our main achievements included the characterization and unraveling of the resistance mechanism conferred by $Yr36$ ($WKS1$ gene), as well as the discovery that $WKS2$-$Ae$ paralog from $A. comosa$, which is lacking the transposon element in intron 10, is fully functional in wheat background.

The characterization of the molecular basis underlying the resistance mechanism conferred by $Yr36$ ($WKS1$) is based mainly on the finding that $WKS1.1$ is inducing programmed cell death by suppressing ascorbate peroxidase (APX) activity and reducing the ability of the cell to detoxify reactive oxygen. The decrease of APX activity reduces the ability of the plant to detoxify the reactive $H_2O_2$ and is the possible mechanism underlying the accelerated cell death observed in the transgenic plants overexpressing $WKS1.1$ and in the regions surrounding a stripe rust infection in the wheat plants carrying the natural $WKS1.1$ gene.

Furthermore, characterization of the epistatic interactions of $Yr36$ and $Yr18$ demonstrated that these two genes have additive effects and can therefore be combined to increase partial resistance to this devastating pathogen of wheat. A similar additive effect was found when combining $WKS1$ from $T. dicoccoides$ and $WKS2$-$Ae$ from $Ae. comosa$, into a single wheat background.

The deciphering and molecular characterization of the resistance mechanism conferred by $Yr36$ ($WKS1$) was based on the following lines of evidence accumulated in the current study:

1. We have identified the lipid ligand of the START domain and characterized the effect of truncations of the START domain on lipid binding;
2. We have determined the location of the splicing variant $WKS1.1$ within the chloroplast.
3. We have determined the cellular localization of splicing variant $WKS1.2$.
4. We have determined that $WKS1.1$ alternative splicing form is the one that confers disease resistance against stripe rust. None of the transgenic wheat lines with $WKS1.2$ cDNA show any resistance to strip rust.
5. We have demonstrated that the chlorosis process of $WKS1$ over-expressing plants exhibited larger $H_2O_2$ staining spots detected across the transgenic leaves.
6. We have demonstrated that $WKS1.1$ mature protein in the chloroplast is able to phosphorylate $TmtAPX$ protein in vivo. The phosphorylation of $TmtAPX$ by $WKS1.1$ protein is proposed as the mechanism of $WKS1$ induction of plant cell death.
In summary, our results suggest the following model: the attack of the stripe rust pathogen increases the proportion of \textit{WKS1.1} protein relative to \textit{WKS1.2}. This increases the amount of \textit{WKS1} protein that enters the chloroplast. In the chloroplast, the mature form of \textit{WKS1.1} protein binds and phosphorylates the APX protein, resulting in reduced APX activity and reduced ability to detoxify the reactive oxygen generated by photosynthesis. The accumulation of reactive oxygen accelerates cell death. This model is consistent with our early finding that \textit{WKS1.1} is more effective at higher temperatures, where ROS levels are higher.

The second part of the project was focused on the characterization of \textit{WKS2}, a non-functional paralog of \textit{WKS1}, which has a retrotransposon insertion close to the alternative splicing site in wild emmer wheat, and to compare it with the structure and function of \textit{WKS2-Ae} derived from \textit{Ae. Comosa}, which is lacking a retrotransposon insertion in intron 10.

The comparison of \textit{TdWKS2} from \textit{T. dicoccoides} with \textit{AcWKS2} derived from \textit{Ae. comosa} yielded interesting results. A comparison of the alternative splicing variants in the \textit{TdWKS2} wheat copy (with the intron10 TE insertion) with the alternative splicing forms of \textit{AcWKS2} in \textit{Aegilops comosa} (no transposon insertion in intron 10) revealed different alternative splicing patterns, regardless of the presence of the retrotransposon insertion. \textit{WKS2} gene in tetraploid wheat RSL65 (\textit{T. dicoccoides} copy with TE) showed three transcript variants, while \textit{AcWKS2} from \textit{Aegilops comosa} (without TE) showed 8 splice variants. However, in both cases, only TV1 variant provided a full length transcript with all 11 exons, including the Kinase and a complete START domain.

To test whether the incorporation of the \textit{AcWKS2} can confer resistance to stripe rust in wheat, we transformed the susceptible common wheat variety Bobwhite with a construct that includes the full length \textit{AcWKS2} cDNA sequence. Two independent transgenic events harboring the full-length of \textit{AcWKS2} cDNA showed partial resistance to stripe rust, similar to the response conferred by \textit{TdWKS1}. Quantitative PCR indicated that the \textit{AcWKS2} is expressed 3-5 folds higher than the nonfunctional \textit{WKS2} in RSL65 and that the expression can be up-regulated by higher temperature and PST-inoculation. Furthermore, transgenic Bobwhite lines containing \textit{WKS1} were crossed with \textit{AcWKS2} Bobwhite transgenes. Plants with \textit{WKS1}, \textit{AcWKS2}, \textit{WKS1×AcWKS2} (F1), or no transgene were compared for the levels of resistance to stripe rust. Our results indicate that plants with \textit{WKS1×WKS2-Ae} showed enhanced resistance to stripe rust than the plants with one gene alone.

These achievements may have a broad impact on wheat breeding efforts attempting to protect wheat yields against one of the most devastating wheat pathogen.

Appendix G6b
Description of the cooperation:

This project is a natural continuation of our collaboration during our previous BARD grant (US-4024-07) that resulted in the positional cloning of Yr36 (WKS1), which is the first partial stripe rust resistance gene in wheat to be cloned. In the current study we have focused our efforts on the mechanism of resistance conferred by this unique gene, composed of a START and a kinase domain. While the team in Davis focused their efforts on the characterization of WKS1, the group in Haifa concentrated their efforts on studying WKS2, the homolog of WKS1. Dr. Ann Blechl transformed WKS2 construct derived from A. comosa WKS2 into susceptible wheat cultivar, Bobwhite. Prof. Fahima spent a Sabbatical year (August 2010-July 2011) at UC Davis, therefore both PIs had plenty of opportunities to meet and discuss the project, and to visit the field plots at UC Davis experimental fields. Later on, Prof. Fahima and Prof. Dubcovsky have met during the International Plant and Animal Genome Conference held in San Diego California (January 2012 and January 2013) as well as in the International Wheat Genetic Symposium held in Yokohama, Japan (September 2013), and discussed the collaboration and the obtained results and planned all of the joint research activities of the US and Israeli groups participating in this project. In addition to that, the PIs and CI coordinated the activities and exchange results via regular email communication and Skype conversations.

The experimental part for the mechanism of WKS1 induction of cell death has been completed. We are currently writing of a manuscript to be submitted to Plant Cell describing the results described in this report. Several additional manuscripts are expected to be submitted in the coming year that will describe the allelic diversity of WKS1 in wild emmer wheat populations in Israel, the splicing variants of WKS2 from Ae. Comosa, as well as the functional validation of WKS2 from Ae. Comosa in wheat background using a transgenic approach.
Appendix 1: Patents and Publications:


**Abstracts presented in conferences:**


8. **Fahima T.** 2012. Genomic studies of agricultural traits in wild emmer wheat. Pintus Memorial Lectures on "Wheat Crop Research: Past and Present" at the Institute of Plant Sciences and Genetics, the Faculty of Agriculture of The Hebrew University, Rehovot, Israel, 27 October 2012. (Invited speaker).

Appendix 2: Unpublished Results

We report here on the molecular characterization of the resistance mechanism conferred by Yr36 (WKS1) by determining the lipid ligand of the START domain, and evaluating the effect of available mutants and alternative splicing variants on lipid binding, chloroplast localization, and kinase activity. Yr36 (WKS1) was resistant to rust and showed necrosis during pathogen infection. Six forms of transcript variants were found in WKS1. The form of WKS1.1 increased during temperature increase and rust infection while other forms decreased their expression levels during rust infection (Fu et al. 2009). Investigated rust growth on transgenic wheat with the cDNA of WKS1.1 or WKS1.2 confirmed that transgenic wheat lines with WKS1.1 cDNA have resistance to strip rust. None of the transgenic wheat lines with WKS1.2 cDNA show any resistance to strip rust demonstrating that the WKS1.1 alternative splicing form is the one that confers disease resistance (Fig. 1). Hydrogen peroxide, \( \text{H}_2\text{O}_2 \), an important reactive oxygen species, has been broadly reported during programmed cell death (Levine et al. 1994). To understand the chlorosis process of WKS1 over-expressing plants better, we stained the leaves of transgenic plants and that of non-transgenic Bobwhite control. Larger \( \text{H}_2\text{O}_2 \) staining spots were detected across the transgenic leaves and some very strong staining areas were even found in almost 1/3 to 1/2 of the whole leaves. In control plant leaves, \( \text{H}_2\text{O}_2 \) staining was restricted only to a small range near leaf tip (Fig. 2).

To establish the sub-cellular localization of WKS1.1 \textit{in vivo}, transgenic wheat plants expressing a fusion between the coding sequence of WKS1.1 and GFP were generated. Confocal microscopy of mesophyll cells from these transgenic plants revealed that GFP fluorescence coincided with chloroplast auto-fluorescence, suggesting that WKS1.1 is localized to the chloroplast. We further extracted the intact chloroplast from the transgenic plants expressing WKS1.1-GFP fusion and then checked GFP signal by Western Blot. A WKS1.1-GFP specific signal was detected in the chloroplast fraction, indicating that the GFP fluorescence in WKS1.1-GFP transgenic plants comes from the fusion protein. To validate these results and to establish the precise localization of WKS1.1 within the chloroplasts, the full length protein was subject to \textit{in vitro} chloroplast import assays. The \textit{in vitro} translation of WKS1.1 resulted in the formation of a 73 kDa protein which represents the precursor form of WKS1.1 (Fig. 3). After chloroplast import, both precursor and mature (truncated) forms of WKS1.1 (upper and lower arrows, respectively) are present in the import reactions, and remain largely associated with the membrane fraction.
after Na₂CO₃ treatment (Lane 4). However, only the mature form of WKS1.1 (Lane 6, upper arrow) is thermolysin-resistant, indicating that mature WKS1.1 is imported into the organelle. This process involves the concomitant loss of ~6 kDa, which most likely represents the signal peptide present in the N-terminal domain of WKS1.1. Finally, WKS1.1 found in the pellet fraction was solubilized by treatment of the pellet with Triton X-100 (Lane 7), confirming that the WKS1.1 protein found in the pellet is a membrane-associated protein (Fig. 3). WKS1.2 encodes for a protein which lacks the last 18 amino-acids of the START domain, but does not result in PST resistance in the transgenic plants (Fig. 1E). Interestingly, during in vitro chloroplast import assays, although WKS1.2 remains largely associated with the membrane fraction after Na₂CO₃ treatment, however, WKS1.2 is thermolysin-sensitive, indicating that WKS1.2 cannot be imported into the chloroplasts. We further analyzed the chloroplast localization of WKS1.1 with transgenic plants over-expression TAP-WKS1.1. After extracting intact chloroplast, we treated the intact chloroplast with trypsin, which is able to digest proteins attached to the outside of chloroplast and those between outer and inner membranes of the chloroplast double layer membrane. After digestion, the TAP-WKS1.1 fusion protein could still be detected in the chloroplast samples although the signal intensity is reduced. The existence of TAP-WKS1.1 fusion protein indicates that WKS1.1 protein could enter wheat chloroplast in the transgenic plants and that the truncation occurs in the C terminus of WKS1.1 protein. We also tested the chloroplast protein of WKS1.1-GFP in transgenic wheat. WKS1.1-GFP fusion protein is clearly detected in intact chloroplasts, however, trypsin digestion eliminates the fusion protein signal, indicating that WKS1.1-GFP fusion protein is only attach outside of chloroplast in transgenic wheat. We further did an in vitro transportation experiment using pea chloroplast and confirmed that in vitro translated WKS1.1-GFP fusion protein attached to but could not enter chloroplast.

In order to establish if the START domain of WKS1.1 and WKS1.2 was indeed capable of binding a lipid or lipids, the binding activity of both proteins to a range of lipids found in biological membranes was experimentally tested. The results revealed that WKS1.2 possesses a differential affinity towards several PIPs to which WKS1.1 either cannot bind or binds with low affinity (Fig. 4). WKS1.2 shows a stronger affinity towards PI (3, 5) P₂ and P₃ (3, 4, 5), which have been implicated in cell death regulation in animals (Stambolic et al. 1998). We developed a cDNA yeast two-hybrid library of stripe rust infected wheat leaves and performed two screens of the library using either the full length WKS1 or the kinase-linker (KI) construct as baits. An APX gene was identified in both the full length WKS1 and KI two-hybrid screens. Since this gene encodes a protein localized in the chloroplast which is known to be
involved in the regulation of oxidative stress and controls the production of H$_2$O$_2$ in plant cells, it was prioritized for further characterization. The identified APX is identical to published thylakoidal APX genes in wheat. To validate the interaction between WKS1.1 and the TmtAPX protein, we carried out co-immunoprecipitation (CoIP) experiment with transgenic wheat over-expressing TAP-WKS1.1 fusion protein. A specific band of APX was clearly detected from the pull-downed samples from TAP-WKS1.1 over-expressing plants. The corresponding band, however, was not detected from samples of prepared from non-transgenic control plants (Fig. 5). Thus, both the yeast data and in vivo CoIP data support that WKS1.1 interacts with TmtAPX, a key enzyme that controls the production of H$_2$O$_2$ in plant cell indicating that TmtAPX may be the intermediate step between WKS1.1 protein function and necrosis phenotype.

To test if WKS1 phosphorylates APX, we searched the full length coding regions of APX gene from wheat. We cloned APX gene from *Triticum monococcum*, TmtAPX1, and used this gene as a model for APX genes in different genomes to study the consequences of WKS1.1-APX interaction. We first investigated the biochemical characteristics of TmtAPX protein. The recombinant protein of TmtAPX prepared from *E. coli* has a wide pH range from 5.0 to 8.0 with an optimum pH at 6.0. The activity of TmtAPX protein was significantly increased by 5 mM MgCl$_2$ but greatly reduced by Ca$^{2+}$, Fe$^{3+}$ and almost totally inhibited by DTT. At optimum reaction conditions, TmtAPX has a $V_{\text{max}}$ of 7.31~7.47 nmole ascorbate acid (ASA) per minute per µg protein and $K_m$ of 6.56±0.72 and 0.70±0.12 mM towards H$_2$O$_2$ and ASA, respectively. We prepared recombinant WKS1.1 protein by in vitro transcription and translation using the wheat germ extracts system. We mixed TmtAPX protein with WKS1.1 protein or GST as negative control and then add ATP to trigger the kinase activity. After 3 hours of reaction, the TmtAPX protein showed clear gel retardation in the sample with WKS1.1 reaction activated by ATP. In the samples using GST instead of WKS1.1 or containing WKS1.1 without ATP, however, this phenomenon was not observed (Fig. 6). The gel retardation showed that WKS1.1 protein function modified TmtAPX and increased its molecular mass. To further study the modification of TmtAPX protein by WKS1.1 reaction, we quantified the phosphorylated peptide after the kinase reaction. We pelleted the total protein after the kinase reaction with or without ATP with cold acetone. The phosphorylated protein was then stained with a fluorescent dye that specifically recognizes phosphorylated peptides. After pelleting protein again and removing excess dye with methanol, the fluorescence bound on the protein was quantified with a fluorescence plate reader. The fluorescence on the samples with ATP has significantly higher reading than the sample without ATP. This data, together with the gel retardation resulted of TmtAPX after WKS1.1 reaction,
showed that TmtAPX was phosphorylated by WKS1.1. To address the consequence of TmtAPX modification by WKS1.1 protein, equal amount of TmtAPX recombinant protein was mixed with GST/ATP, WKS/ATP or WKS1. The activity of TmtAPX was quantified after the 3 hour long modification process. Samples with GST/ATP or those with WKS1.1 protein had similar APX specific activity with no significant changes (1.54 Vs 1.57 n mole/ug protein/min, t-test p value= 0.906), indicating that the physical binding of WKS1.1 with TmtAPX has little effect on APX activity (Fig. 6). The samples with WKS1.1 protein and ATP, however, had significantly reduced activities by about 40%. In summary, the APX activity of the recombinant protein of TmtAPX could be reduced by WKS1.1 protein in vitro. The decrease of APX activity reduces the ability of the plant to detoxify the reactive H₂O₂ and is the possible mechanism underlying the accelerated cell death observed in the transgenic plants overexpressing WKS1.1 and in the regions surrounding a stripe rust infection in the wheat plants carrying the natural WKS1.1 gene.

To study the phosphorylation ability of WKS1.1 mature protein, we tests different truncated forms of WKS1.1 protein. We used the WKS1.1 kinase and kinase-linker partial proteins as models of C terminus truncation to test the possible phosphorylation ability using recombinant TmtAPX protein as substrate. The two truncated proteins produced significant increases of phospho fluorescence and protein retardation in a specific gel with Phos-tag which binds phosphorylated protein (Fig. 7). These results indicate that mature form of WKS1.1 with the 6 kDa truncation should be able to phosphorylate TmtAPX. We then extracted intact chloroplasts from the NP::WKS1 transgenic wheat and analyzed the phosphorylation status of TmtAPX using the Phos-tag gel. Clear gel retardation was observed in APX from samples of transgenic intact chloroplast compared with non-transgenic Bob White control (Fig. 7). This data supports that WKS1.1 mature protein in the chloroplast is capable to phosphorylate TmtAPX protein in vivo.

The phosphorylation of TmtAPX by WKS1.1 protein is proposed as the mechanism of WKS1 induction of plant cell death. To verify that WKS1.1 and TmtAPX work together to regulate cell death in plant, we combined the WKS1.1 over-expression transgene with an APX deletion previously described in wheat. The APX 6B deletion mutant has a 40% reduction in total thylakoid APX activity but showed no oxidative stress probably due to the presence of redundant APC genes in the 6A and 6D chromosomes (Danna et al. 2003). We crossed NP: TAP-WKS1.1 over-expression (hereafter TAP-WKS1.1) transgenic plant and APX 6B deletion mutant and analyzed the phenotypes of the near-isogenic offspring. In the T₃ generation plants, TAP-WKS1.1 and APX 6B deletion double mutants were pale green in the majority part of the leaves, similar to those of APX 6B deletion single mutants. Plants carrying both TAP-WKS1.1 and the APX 6B
deletion, showed early senescence in leaf tips, which was not observed when the APX 6B deletion or the TAP-WKS1.1 were present separately (Fig. 8). Furthermore, DAB staining showed strong reactive oxidant species accumulation in the necrosis spots, indicating that the presence of both TAP-WKS1.1 over-expression and the APX 6B deletion further increases the inability of the plant to detoxify ROS (Fig. 8). These results support the hypothesis that the increase in DAB staining in the TAP-WKS1.1 over-expression is mediated by a reduction in APX activity. In summary, our results suggest to following model: the attack of the stripe rust pathogen increases the proportion of WKS1.1 protein relative to WKS1.2. This increases the amount of WKS1 protein that enters the chloroplast. In the chloroplast, the mature form of WKS1.1 protein binds and phosphorylates the TmtAPX protein, resulting in reduced APX activity and reduced ability to detoxify the reactive oxygen generated by photosynthesis. The accumulation of reactive oxygen accelerates cell death. This model is consistent with our early finding that WKS1.1 is more effective at higher temperatures, where ROS levels are higher.

We also completed the characterization of the epistatic interactions of Yr36 and Yr18 and demonstrated that these two genes have additive effects and can therefore be combined to increase partial resistance.

WKS2, a non-functional paralog of WKS1, which has a retrotransposon element (TE) insertion close to the alternative splicing site in cultivated wheat but not in some wild wheat relatives. Detailed characterization of the alternative splicing of WKS2 gene revealed 3 transcript variants in tetraploid wheat RSL65 (T. dicoccoides copy with TE) and 8 variants in wild relatives (WKS2 in Aegilops comosa, designated WKS2-Ae, without TE). The three transcript variants in RSL65 (WKS2.1 to 2.3) were revealed by cloning and sequencing of 50 WKS2 cDNAs. WKS2.1 encodes a complete WKS2 protein, whereas the other two encode truncated WKS2 proteins caused by premature stop code (Fig. 9). Cloning and sequencing of 48 WKS2-Ae cDNA clones in Aegilops comosa (no transposon inerstion in intron10) revealed eight alternative transcript variants (WKS-Ae2.1 to 2.8). WKS-Ae2.1 encodes a complete WKS2-Ae protein, whereas the other seven (WKS-Ae 2.2 to WKS-Ae 2.8) lack exon11 and encode proteins with truncated START domains (Fig. 10). Comparisons between WKS-Ae 2.1 and WKS-Ae 2.2-8 transcript levels in heading, anthesis, 3 DAA (days after heading), 6DAA and 9DAA plants showed higher values of WKS-Ae 2.1 relative to WKS-Ae 2.2-8 in all of the stages. The differences are not significant at heading and 9DAA stages and significant at anthesis (P<0.01), 3DAA (P<0.05) and 6DAA (P<0.01) stages (Fig. 11).
To test if the incorporation of the \textit{WKS2-Ae} can confer resistance to stripe rust to wheat, we transformed the susceptible common wheat variety Bobwhite with the PWKS2-Ae plasmid, which includes the full length of \textit{WKS2-Ae} cDNA sequence. Two transgenic positive lines K69-162-13, K69-34L-8 with the full-length of \textit{WKS2-Ae} cDNA sequence identified by PCR specific primers and sequencing, shows increased resistance to stripe rust (Fig. 12, 13 and 14). Quantitative PCR indicated that the \textit{WKS2-Ae} is expressed about 3-5 folds higher than the nonfunctional \textit{WKS2} in RSL65 (Fig. 14) and the expression can be up-regulated by higher temperature and PST-inoculation (Fig. 15). Furthermore, transgenic Bobwhite lines containing \textit{WKS1} were crossed with \textit{WKS2-Ae} Bobwhite transgenes. Plants with \textit{WKS1}, \textit{WKS2-Ae}, \textit{WKS1×WKS2-Ae} (F$_1$), or no transgene were compared for the levels of resistance to stripe rust. Percentage of leaf area covering with pustules was categorized by "pd" program (Fu et al. 2009, Fig. 16). The results from three averaged replicates indicate that plants with \textit{WKS1×WKS2-Ae} shows enhanced resistance to the stripe rust than the plants with one gene alone (Fig. 17).

We screened 44 different \textit{Hordeum} collections for functional \textit{WKS1} with specific primers, no \textit{WKS1} positive accessions were found. In order to determine if wheat \textit{WKS1} improves resistance of barley to stripe rust, we transferred the stripe rust susceptible barley variety Golden Promise with the \textit{WKS1} construct used before (Fu et al. 2009), which conferred resistance to the susceptible wheat variety Bobwhite. The transgenic T$_1$ plants were detected by \textit{WKS1} specific primers (Fig. 18). No products were amplified in transgenic line UB1-1 and this line can be considered as the transgenic negative control. All the T$_1$ plants with or without \textit{WKS1} were grew in the greenhouse and the T$_2$ seeds were obtained. However, we still do not know the effect of wheat \textit{WKS1} on stripe rust resistance in transgenic barley because we did not find the \textit{Puccinia striiformis} \textit{Hordeum} isolates which can infect on barley variety golden promise in Israel and it is complicate and difficult to import the PSH isolates from abroad. Therefore, we will continue this work in the future when we have the infected PSH isolates.
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Fig. 1. WKS1.1 and WKS1.2 transgenic wheat response to PST infection.
A) Glasgow: susceptible response.
B) UC1041: susceptible response.
C) UC1041+Yr36: natural Yr36 partial resistance response.
D) Transgenic NP::WKS1.2: susceptible response.
E) Transgenic NP::WKS1.1 (event 29.4.6.): partial resistance response.
F) Transgenic NP::WKS1.1 (event 37.3): partial resistance response.

Fig. 2. Diaminobenzidine (DAB) staining of the second leaves to show the reactive oxidants accumulation in WKS1 over-expressing plants.

In situ detection of hydrogen peroxide is presented in mature wheat leaves by staining with 3,3'-diaminobenzidine (DAB) using standard protocol. DAB is oxidized by hydrogen peroxide in the presence of some haem-containing proteins, such as peroxidases, to generate a dark brown precipitate.
Fig. 3. Chloroplast transportation of WKS1.1 and WKS1.2 in vitro translated protein.

Fig. 4. PIP binding kinetics of WKS1.1 and WKS1.2.
Fig. 5. Co-immunoprecipitation of *TmAPX* with TAP-*WKS1.1* from transgenic wheat.

Fig. 6. *TmAPX* gel retardation (left) and activity decrease (right) after *WKS1.1* reaction.

Fig. 7. *TmAPX* phosphorylation by truncated *WKS1* proteins (top) and *in planta* phosphorylation in intact chloroplasts of *WKS1* transgenic plants (bottom).
Fig. 8. Accelerated cell death in WKS1.1 over-expression and APX 6B deletion double mutant.

Fig. 9. WKS2 alternative transcript variants
WKS2 transcript variants in wheat RSL65 (WKS2 with retrotransposon insertion)
TV1 variant with 11 exons including the Kinase and a complete START domain.
TV2 transcripts with 11 exons which have intron 4 retention that changes the reading frame and generates a premature stop codon in intron4.
TV3 transcript variant do not include exon 11 and have an 8bp alternative 5' splice in intron4 which change the reading frame and premature stop code in exon6. Besides, it also has a premature splicing site in exon7 (56bp before the GT splice site).
**Fig. 10. WKS2-Ae alternative transcript variants**

*WKS2-Ae* transcript variants in amphiploid accession #3402 (*WKS2* derived from *Aegilops comosa*)

TV1 is the only variant with 11 exons coding for a complete START domain.

TV2-8 transcript variants do not include exon 11 and have a premature stop codon which generated truncate protein.

TV2 transcripts continue beyond the GT splicing site after exon 10 until a stop codon 153-bp after this splicing site.

TV3 transcripts have intron 4, intron 5, intron 6 and intron 8 retention, which changes the reading frame and generates a premature stop codon in exon 4.

TV4 transcripts have intron 4 and intron 5 retention, which changes the reading frame and generates a premature stop codon in exon 4.

TV5 transcripts do not include the second exon (marked in red, exon skipping). This difference generates a change in reading frame and a premature stop codon in exon 4. Besides, it also has intron 4, intron 5 retention and 8bp alternative 3' splice in intron 8.

TV6 transcripts do not include the second exon (marked in red, exon skipping). This difference generates a change in reading frame and a premature stop codon in exon 4. Besides, it also has a premature splicing site in exon 5 (24bp before the GT splice site).

TV7 transcripts do not include the second exon (marked in red, exon skipping). This difference generates a change in reading frame and a premature stop codon in exon 4. Besides, it also has intron 4, intron 5 and intron 6 retention.

TV8 transcripts do not include the second exon (marked in red, exon skipping). This difference generates a change in reading frame and a premature stop codon in exon 4.
Fig. 11. The expression profiles and relative transcript quantity of transcript variants WKS-Ae 2.1 and WKS2.2-8 during heading, anthesis, 3DAA, 6DAA and 9DAA stages detected by qRT-PCR. Each data point is the average of 3 biological replicates ± SE of the mean. *P<0.05, **P<0.01.

Fig. 12. Resistance or susceptible response on leaves after inoculated with race PST-38E134. Scale bar 5mm. (A) RSL65 with partial stripe rust resistance gene WKS1. (B) WKS1 independent transgenic event 17a in hexaploid wheat variety Bobwhite (BW). (C) WKS2-Ae independent transgenic event K69-162-13C in Bobwhite. (D) Untransformed variety Bobwhite.
Fig. 13. WKS2-Ae transformation vector and molecular characterization of transgenic wheat. 
(A) In transformation vector PC221, the WKS2-Ae gene was driven by Ubi promoter and terminated by the Agrobacterium tumefaciens nopaline synthase gene (Tnos). The Bar gene and Hygromycin gene were used as selectable markers to screen putatively transformed T0 plants. (B) PCR pattern of WKS2-Ae transgenic plants K69-162-13C using the transgene specific primers P2F/P2R (B, line 1), P1F/P1R (B, line 2). (C) PCR pattern of WKS2-Ae transgenic plants K69-34L-8 using the transgene specific primers P2F/P2R (B, line 1), P1F/P1R (B, line 2). 1-10, ten independent transgene plants. M, marker.

Fig. 14. WKS2-Ae expression levels and resistance phenotype in transgenic wheat plants. 
A: Average WKS2-Ae expression levels (±SEM) in independent transgenic events K69-34L-8 (four plants) and K69-162-13C (three plants) were determined by real-time (RT) PCR and compared with RSL65. Susceptible lines LDN, Bobwhite and transgenic events K69-34L-3 do not contain WKS genes were included as negative controls. B: Leaf phenotypes (S, susceptible; R, resistant). Scale bar 1mm.
Fig. 15. Effect of temperature and PST inoculation on transcript levels of WKS2-Ae in transgenic wheat plants. The effect of high (10/25°C) and low (10/15°C) temperature on transcript levels of WKS2-Ae was determined by Q-PCR. Leaf samples from K69-34L-8 plants were collected 3, 9 and 16 days post inoculation (DPI) with race PST-38E134. PST-inoculated plants are indicated by solid color and non-inoculated controls by stripes. Each data point is the average of 3 biological replicates ± SE of the mean. *P<0.05, **P<0.01, ***P<0.001; NS, not significant.
**Fig. 16.** Visual display of digital quantification of the percentage of leaf area covered with *Puccinia striiformis* pustules. (A, C and E) Images of 2-cm segments of wheat leaves. (B, D and F) Each pixel in A, C and E was categorized by "pd" program as either leaf (green), *P. striiformis* (red) or background (black). (A and B) Susceptible negative 34L-3 with pustules covering 14.65% of the surface area in the segment shown in A. Transgenic resistant 162-13c (WKS2) and 17a4 (WKS1) with pustules only on 2.39% and 1.22% of segments shown in C and E, respectively. Bar= 2mm.
Fig. 17. PCR markers used to detect \( WKS1 \times WKS2\_Ae \) F1 plants and the leaves stripe rust disease severity analysis by “pd” program.

A. The primer pair \( WKS\_540-544 \) specific for the START domain amplifies products of 871-bp from \( WKS1 \), 537-bp from \( WKS2 \) and 171-bp from \( WKS2\_Ae \). These primers require an initial touchdown with a decrease of 0.5°C per cycle from 70 to 65 °C (60 s per cycle).

Two pairs of primers, each specific for one of the \( WKS \) genes, were used to determine the presence of the \( WKS \) genes (B, C). B, \( WKS1\_150-620 \) amplifies a product of 857bp when \( WKS1 \) is present and no product in its absence. C, \( WKS2\_652-648 \) amplifies a product of 979-bp when \( WKS2\_Ae \) is present and no product when it is absent. These primers require an initial touchdown with a decrease of 0.5 °C per cycle from 64 to 60 °C (60 s per cycle). D, Average leaf area covered with \( Puccinia striiformis \) pustules. Infected wheat leaves were categorized by the image analysis program “pd”. The percentage of pustules covering on a 2-cm segment leaf area was used to count the strip rust disease severity. BW, susceptible Bobwhite; 34L-3, transgenic events without \( WKS2\_Ae \) (negative control); \( WKS2, WKS2\_Ae \) in transgenic line K69-34L-8; \( WKS1, WKS1 \) in transgenic line 26B15; \( WKS1 \& WKS2, 26B15\times34L-8 \). Averages are of three replications.

Fig. 18. PCR markers used to detect \( WKS1 \) in transgenic barley lines.

A, Hyg F/R; B, \( WKS1\_150-620 \). A, The primer pair specific for the hygromycin gene (amplifies products around 600bp). B, \( WKS1 \) specific primer \( WKS1\_150-620 \), used to determine the presence of the \( WKS1 \) gene, amplifies a product of 857bp when \( WKS1 \) is present and no product in its absence. This primer requires an initial touchdown with a decrease of 0.5 °C per cycle from 64 to 60 °C (60 s per cycle). UB, constructs: pB214 \( WKS1 \) over-expression with Ubiquitin promoter; NP, constructs: pB203 \( WKS1 \) driven by native promoter.
References:


